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STIM1 mediates multiple signalling pathways in neuronal growth cones

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Mitchell CB, Gasperini RJ, Small DH, and Foa L. (2012) STIM1 is necessary for store-operated calcium entry in turning growth cones. *J Neurochem*, **122**, 1155-1166.

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Statement of ethical conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

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Abstract

Calcium is an intracellular second messenger that is vital for normal neuronal function. The maintenance of calcium homeostasis is critical for healthy neuronal function, and disruption in calcium homeostasis has been implicated in diseases such as epilepsy and Alzheimer's disease. In developing neurons, calcium signalling regulates the precise wiring of neurons, in a process known as axon guidance. Axon guidance is extremely important in the normal healthy development of the nervous system. Aberrant axon guidance is highly associated with several neurodevelopmental disorders including autism and mental retardation syndromes such as fragile-X syndrome. Axons navigate the environment by a dynamic navigational structure located at the distal tip of an extending axon, known as a growth cone. Cytosolic calcium is crucial in mediating growth cone navigation. Correct understanding of the signalling mechanisms that regulate cytosolic calcium is key to understanding normal growth cone function.

This thesis focuses on the molecular mechanisms that regulate a vital source of calcium within growth cones, the endoplasmic reticulum (ER). Little is known about the function of the ER within growth cones. Stromal Interaction Molecule 1 (STIM1) is a calcium sensing protein in the ER membrane, which interacts with Orai proteins in the plasma membrane to initiate store-operated calcium entry (SOCE) and refill depleted intracellular calcium stores. The central hypothesis of this thesis is that STIM1 is necessary for SOCE in neuronal growth cones, and is required for axon guidance.

The results presented within this thesis demonstrate the presence and function of STIM1-mediated processes within the developing nervous system. This thesis has utilised primary cell culture of embryonic dorsal root ganglia neurons and immunocytochemistry to investigate the presence and localisation of STIM1 within developing growth cones. STIM1, along with its binding partners Orai1 and Orai2 reside in two different localisation patterns within growth cones; active (punctate) and inactive (diffuse). Depletion of calcium stores resulted in the activation of STIM1 within growth cones, increasing the number of growth cones displaying punctate STIM1 protein distribution. Calcium depletion also increased colocalisation between STIM1 and Orai1. Furthermore, STIM1 localisation appeared to be biased towards the turning side of the growth cone, in response to a calcium-dependent guidance cue. These data suggest that STIM1 and the Orai proteins are dynamic proteins that function in the regulation of calcium within growth cones.

While immunocytochemistry data suggested that STIM1 was functional within growth cones, a target morpholino approach was used to determine if STIM1 was necessary for growth cone function. A reduction of endogenous STIM1 reversed turning towards BDNF and netrin-1, and demonstrated that STIM1-mediated SOCE was necessary for BDNF signalling in growth cones. Unexpectedly, a reduction in STIM1 abolished turning away from Sema-3a in a manner independent of SOCE. In a growth cone collapse assay, STIM1 was also found to be necessary for Sema-3a-induced collapse, suggesting that STIM1 is implicated in multiple Sema-3a signalling pathways. This knockdown approach clearly demonstrates the necessity of STIM1 function for normal growth cone turning.

While the main function of STIM1 is thought to be the activation of Orai proteins, and subsequent activation of SOCE, STIM1 has been shown to interact with other signalling proteins, including the second messenger cAMP, in a process termed store-operated cAMP signalling. This study utilised cAMP analogues to determine if store-operated cAMP signalling was functional within growth cones. Upon the activation of cAMP, repulsive turning away from Sema-3a was restored in growth cones with reduced levels of STIM1. Sema-3a collapse was also prevented upon addition of cAMP agonists in control growth cones, but not restored in STIM1 morphants. Similar results were achieved with cGMP agonists. These data suggest that STIM1 mediates cyclic nucleotide signalling within growth cones. Furthermore, STIM1 has also recently been implicated in the reciprocal control of L-type voltage-gated calcium channels (VGCCs) and Orai proteins. While L-type VGCCs are important in mature neurons, there is conflicting data in the literature as to their role in axon guidance. This study investigated whether there was a potential interaction between STIM1 and L-type VGCCs in growth cones, and found that if there is an interaction, it is not essential for growth cone turning, but may be required for axon extension.

These results indicate a number of novel findings: Firstly, that STIM1 mediates growth cone navigation in response to both calcium-dependent and -independent guidance cues. Secondly, that STIM1 is required for Sema-3a signalling. Thirdly, that STIM1 mediates cyclic nucleotide signalling pathways within growth cones, and likely does not interact with

L-type VGCCs for growth cone navigation. In conclusion, this thesis has significantly added to the understanding of the regulation of the calcium signalling pathways that are crucial for normal growth cone guidance, enhancing our understanding of growth cone navigation, and in particular the regulation of the calcium signalling pathways that are crucial for normal growth cone guidance. These findings add to the pool of knowledge of how growth cones function and regulate calcium, which is crucial for normal neuronal health within development.

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Abbreviations List

| | |
|----------------------------------|-------------------------------------|
| [Ca ²⁺] _i | Intracellular calcium concentration |
| 6-Phe-cAMPS | Sp-6-Phe-cAMPS |
| 8-Me-cAMPS | 8-pCPT-2'-O-Me-cAMPS |
| AC | Adenylate cyclase |
| Ach | Acetylcholine |
| ATP | Adenosine triphosphate |
| ATPase | Adenosine triphosphatase |
| BDNF | Brain derived neurotrophic factor |
| CAD | CRAC activation domain |
| CaMKII | Calcium/calmodulin kinase II |
| cAMP | Cyclic adenosine monophosphate |
| CaN | Calcineurin |
| cGMP | Cyclic guanosine monophosphate |
| CIF | Calcium Influx Factor |
| CNS | Central nervous system |
| CRAC | Calcium-release activated calcium |
| CICR | Calcium-induced calcium release |
| DCC | Deleted in Colon Cancer |
| DRG | Dorsal root ganglia |
| EDTA | Ethylenediaminetetraacetic acid |

| | |
|------------|---------------------------------------------|
| EGTA | Ethylene glycol tetraacetic acid |
| Epac | Exchange protein directly activated by cAMP |
| ER | Endoplasmic reticulum |
| ER-PM | Endoplasmic reticulum-plasma membrane |
| ERM | ezrin/radixin/moesin |
| f-actin | Filamentous actin |
| FLIP | Focal laser-induced photolysis |
| GTP | Guanosine triphosphate |
| HBSS | Hank's Buffered Salt Solution |
| I_{CRAC} | CRAC current |
| IP_3 | Inositol trisphosphate |
| IP_3R | IP_3 receptor |
| IR | Immunoreactivity |
| MAG | Myelin-associated glycoprotein |
| MAPK | Mitogen-activated protein kinase |
| mRNA | messenger RNA |
| NaCl | Sodium Chloride |
| NFAT | Nuclear Factor of Activated T cells |
| NGF | Nerve Growth Factor |
| Npn | Neuropilin |
| NT-3 | Neurotrophin 3 |

| | |
|--------------------|--------------------------------------------|
| NT-4/5 | Neurotrophin 4/5 |
| P75 ^{ntr} | p75 neurotrophin receptor |
| PBS | Phosphate buffered saline |
| PDE | Phosphodiesterase isozyme |
| PI3-K | Phosphoinositide 3-Kinase |
| PIP ₂ | Phosphatidylinositol 4,5-bisphosphate |
| PKA | Protein Kinase A |
| PKG | Protein Kinase G |
| PLC | Phospholipase C |
| PTEN | Phosphatase and tensin homolog |
| PVDF | polyvinylidene difluoride |
| Rac1 | Ras-related C3 botulinum toxin substrate 1 |
| RhoA | Ras homolog gene family, member A |
| RIPA | Radioimmunoprecipitation assay buffer |
| RNAi | RNA interference |
| ROI | Region of interest |
| RyR | Ryanodine Receptor |
| SAM | Sterile Alpha Motif |
| SCID | Severe Combined Immunodeficiency |
| SDS | Sodium dodecyl sulfate |
| SEM | Standard error of mean |

| | |
|----------------|----------------------------------------|
| Sema-3a | Semaphorin-3a |
| SERCA | Sarco/endoplasmic reticulum |
| SNM | Sensory Neuron Media |
| SOC | Store- operated calcium |
| SOCC | Store- operated calcium channel |
| SOCE | Store-operated calcium entry |
| STIM | Stromal Interaction Molecule |
| Tg | Thapsigargin |
| TrK | Tyrosine Kinase |
| TRP | Transient receptor potential |
| TRPC | Transient receptor potential canonical |
| VGCC | Voltage-gated calcium channel |
| w/w | weight/weight |
| <i>Xenopus</i> | <i>Xenopus laevis</i> |